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14. ABSTRACT

We identified a gene TMS1 that is subject to epigenetic silencing in breast cancer, although the precise role of TMS1 in the pathogenesis of cancer is unknown. We hypothesize that silencing of TMS1 contributes to breast carcinogenesis by allowing cells to bypass apoptosis, and consequently may cause cells to be resistant to chemotherapy. We created a TMS1 loss-of-function model in MCF7 cells using siRNA, and determined that cells lacking TMS1 expression are approximately 2-fold more resistant to certain apoptotic stimuli. Also, whereas TMS1 is not required for TNFα or TRAIL-induced activation of NF-κB or caspase-8, it promotes caspase-8 activation independently of death receptor stimuli. We also find that TMS1 is upregulated by TNFα in breast epithelial cells, but not in fibroblasts. This induction was blocked by knockdown of RelA/p65, and is not dependent on new protein synthesis or mRNA stability, suggesting a direct effect on TMS1 transcription. Consistent with this, TNFα treatment causes local chromatin alterations at the TMS1 locus. Although previous work has suggested that TMS1 is regulated by p53, we found little impact of DNA damaging agents on TMS1 expression. Upregulation of TMS1 by TNFα and subsequent activation of caspase-8 could function to amplify death receptor-induced apoptosis.

15. SUBJECT TERMS
Apoptosis, tumor suppressor gene, cell biology, gene expression, molecular genetics, chemotherapy and experimental therapeutics

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A. Introduction

Breast and other cancers arise from mutations in tumor suppressor genes and oncogenes that accumulate in normal tissue over time. Aberrant methylation of promoter region CpG islands (dense regions of cytosine-guanine dinucleotides) is associated with gene silencing and serves as an alternative to mutations in the inactivation of tumor suppressor genes in human cancers. Like a mutation, such epigenetic modifications, once acquired, persist in subsequent cell divisions, leading to transformation and ultimately tumor formation. However, contrary to mutations in the DNA sequence itself, tumor suppressor genes that are silenced through epigenetic means remain structurally intact, thus the possibility exists for re-expression of aberrantly-methylated genes as a therapeutic strategy in human cancers.

Our lab has identified a novel, proapoptotic gene named TMS1 (for Target of Methylation-Mediated Silencing 1, also known as ASC or Apoptosis-Associated Speck-Like Protein Containing a Caspase-Recruitment Domain) that is aberrantly methylated and consequently silenced in 40% of primary breast tumors. Subsequent work showing that TMS1 is a target of methylation-mediated silencing in lung cancers, ovarian cancers, melanomas, and glioblastomas suggests that silencing of TMS1 contributes to the pathogenesis of a number of different tumor types. However, at present the consequences of TMS1 silencing and its role in carcinogenesis are not known. The TMS1 protein contains a caspase-recruitment domain and a pyrin domain, which are two protein-protein interaction motifs. These motifs are often found in intracellular signaling molecules involved in the regulation of apoptosis and inflammation. Evidence from overexpression studies performed in our lab indicates that TMS1 drives apoptosis in a caspase 9-dependent manner [1]. Furthermore, it was recently published that TMS1 binds to the Bcl-2 family member Bax and facilitates its translocation to the mitochondrion in response to cytotoxic agents [2]. Consistent with a role in the intrinsic (mitochondrial) cell death pathway, antisense-mediated knockdown of TMS1 protects cells from apoptosis induced by cytotoxic agents [3]. TMS1 has also been implicated in the extrinsic apoptotic pathway, as it binds to and activates caspase-8, the apical caspase induced by death receptors [4]. Recent biochemical data and evidence from knockout mice indicate that TMS1 functions as an adaptor for caspase-1, and that it is necessary for the maturation of proinflammatory cytokines in response to bacterial pathogens in cells of myeloid lineages [5]. Whether it plays a similar, or perhaps additional role in breast cancer cells is not known.

It is our hypothesis that epigenetic silencing of TMS1 contributes to carcinogenesis by allowing breast cancer cells to bypass normal apoptotic cues and, as a consequence, may cause some cancers to be resistant to chemotherapy. There were three specific aims associated with this proposal. The first was to create a model of TMS1 loss-of-function in human breast cancer cells. As reported in the annual summary for reporting period 17 July 2003 – 16 July 2004, this step of the project has been completed, although through alternate means than those originally proposed. The second task of this proposal was to determine whether loss of TMS1 causes resistance of breast cancer cells to anticancer agents and proapoptotic stimuli, and to characterize the resulting apoptotic response using the cell lines created in Task 1. Task 3, which has been initiated ahead of schedule, was to determine the effects of TMS1 loss on anchorage-independent growth potential. In this annual summary report, I will discuss the progress made on Tasks 2 and 3, as well as discuss other novel directions this project has taken, important findings, and reportable outcomes.

B. Body

The overall goal of this proposal was to model the loss of TMS1 seen in breast cancer and to determine the consequences of this silencing on the cellular response to chemotherapy and other proapoptotic stimuli, as well as to examine anchorage-independent growth potential following TMS1 loss. The first task, which was completed over the last reporting period, involved developing a model of TMS1 loss-of-function in human breast cancer cells. This task was achieved not through stable transfection of siRNA (short interfering RNA) targeting TMS1, as originally proposed, but was achieved through optimization of transient siRNA transfection in the breast cancer cell line MCF7, resulting in a virtual 100% loss of TMS1 expression. Using this system I have been able to address Tasks 2 and 3 proposed in my original statement of work, as well as to examine differences in cytokine-induced NF-κB (nuclear factor-kappaB) activity between TMS1 expressing and non-expressing MCF7 cells. In addition, this project has made significant progress in the area of TMS1 regulation in breast epithelial cells. Understanding the regulation of TMS1 in breast epithelial cells may shed light on the function of TMS1 in this cell type, which at present is unknown. In this annual summary report I will discuss the progress made on Tasks 2 and 3 of my proposal, as well as discuss new data focused on the regulation of TMS1 in breast epithelial cells.

The focus of Task 2 was to determine whether loss of TMS1 causes resistance of breast cancer cells to anticancer agents and other proapoptotic stimuli, and to characterize the resulting apoptotic response using a TMS1 loss-of-function model created in Task 1. Using the siRNA-mediated knockdown system created in Task 1, I have shown that MCF7 cells lacking TMS1 expression through transfection of siRNA to TMS1 are approximately two-fold more resistant to apoptosis induced by the death receptor ligands TNFα (tumor necrosis factor alpha) and TRAIL (TNF-related apoptosis inducing ligand), as well as the DNA damaging agents etoposide and mitomycin c, compared to control MCF7 cells as determined by trypan blue staining (Appendix A, Figure 1). This result indicates that silencing of TMS1 in breast and other cancers may allow cancer cells to bypass apoptosis induced by these agents, thereby dampening the therapeutic response and increasing survival of cancer cells.

It is well established that death-receptors induce apoptosis through activation of caspase-8, which is the apical caspase in the death-receptor (extrinsic) apoptotic pathway. Previous work from our lab and others has indicated that TMS1 activates capsase-8 when overexpressed in HEK (human embryonic kidney) 293 cells ([4, 6] and data not shown). Therefore, to further characterize the consequences of TMS1 loss on cell death pathways, we examined caspase-8 activation induced by death-receptors in MCF7 cells transfected with siRNA targeting TMS1 or lamin A/C as a control. siRNA-mediated knockdown of TMS1 expression in MCF7 cells had little impact on TNFα- or TRAIL-induced caspase-8 cleavage and activation, suggesting that TMS1 is dispensable for caspase-8 activation induced by death-receptors in this cell type (Appendix A, Figure 2 and Appendix B, Figures 5c and 5d). However, TMS1 overexpression in MCF7 cells lead to activation of caspase-8 in a dose-dependent manner (Appendix A, Figure 3 and Appendix B, Figure 5e), suggesting that although TMS1 is not necessary for activation of capsase-8 induced by death-receptors, it promotes cleavage of caspase-8 independent of a death-receptor stimulus. Therefore, it is possible that TMS1 can amplify the apoptotic signal induced by death-receptor pathways, and that loss of TMS1 in breast cancer could dampen the apoptotic response to death receptor ligands such as TRAIL and TNFα.

Aside from activating apoptosis, death-receptors can also activate NF-κB signaling. NF-κB is an important regulator of inflammation and apoptosis, and its activity is often dysregulated

in human cancers. Previous reports have indicated that TMS1 can either promote or inhibit NF-κB signaling depending on cell type, the coexpression of specific adaptor proteins and/or TMS1 expression levels [4, 6-8]. To further characterize the function of TMS1, we examined the impact of TMS1 loss on NF-κB activation induced by death receptors. We found that loss of TMS1 expression by transient siRNA transfection in MCF7 cells did not impact NF-κB activity induced by TRAIL or TNFα (Appendix A, Figure 4 and Appendix B, Figures 5a and 5b), suggesting that TMS1 is dispensable for NF-κB activation induced by death-receptor signaling in this cell type.

In the annual summary for the reporting period 17 July 2003 – 16 July 2004, I discussed the novel finding that TNFα is a positive regulator of TMS1 expression in breast epithelial cells. I also reported that the induction of TMS1 by TNFα is mediated at the level of transcription and requires the activity of NF-κB and JNK (jun kinase) signaling pathways. I have made significant progress over the last year characterizing the regulation of TMS1 by TNFα. I have also examined the response of TMS1 to other proapoptotic stimuli. I have determined that TNFα-induced upregulation of TMS1 expression is particular to breast epithelial cells, and is not observed in the normal human diploid fibroblast cell line IMR90 (Appendix A, Figure 5 and Appendix B, Figures 1b and 2c). In addition, contrary to a previous report, I have found that DNA damaging agents that induce stabilization of the transcription factor p53 have little effect on TMS1 expression at either the mRNA or protein level in breast epithelial cells or human fibroblasts (Appendix A, Figure 6 and Appendix B, Figures 1a-2c). Using transient siRNA transfections, I also determined that the RelA/p65 subunit of NF-κB is required for upregulation of TMS1 by TNFα in MCF7 cells, thus further characterizing the involvement of the NF-κB signaling pathway on TMS1 expression following TNFα treatment (Appendix A, Figure 7 and Appendix B, Figures 3e and 3f).

NF-κB and JNK activity are known to stimulate the rapid expression of a number of genes, some of which could themselves be contributing to the TNFα-induced upregulation of TMS1. The timeframe of TMS1 induction following TNFα stimulation, which is not observed until 24 hours after treatment (data not shown), is also suggestive of the involvement of a TNFα-regulated intermediate. I therefore examined the requirement for secondary protein synthesis in the upregulation of TMS1 following TNFα stimulation, and found that blocking new protein synthesis with cyclohexamide does not inhibit TNFα-induced upregulation of TMS1 mRNA (Appendix A, Figures 8a and 8b and Appendix B, Figures 4a and 4b), illustrating that secondary protein synthesis is not required for TMS1 upregulation following TNFα treatment. To further address the mechanism by which TNFα upregulates TMS1, I also examined TMS1 mRNA stability following TNFα treatment, and observed that the half-life of TMS1 mRNA following TNFα stimulation is not significantly altered compared to untreated cells (Appendix A, Figure 8c and Appendix B, Figure 4d).

Taken together, these data suggest that TNFα-induced upregulation of TMS1 is a direct effect occurring at the level of transcription. Consistent with this idea, I have observed that TNFα treatment induces local alterations in chromatin structure at the TMS1 locus as observed by DNase-I hypersensitive site analysis (data not shown). In addition, the DNaseI hypersensitive sites found in TMS1 expressing breast epithelial cells (MCF7 cells) are lacking in the breast epithelial cell line MDA-MB231, which is methylated and silenced at the endogenous TMS1 gene and therefore do not express TMS1 (Appendix A, Figure 9). This is suggestive of a "closed" or otherwise inaccessible chromatin conformation. I have also found that treatment of

this cell line with TNFα or TRAIL does not restore TMS1 expression (data not shown), suggesting that upregulation of TMS1 by cytokine signaling is dependent upon a particular chromatin conformation and that treatment of cells with TNFα or TRAIL is not sufficient to overcome epigenetic silencing of TMS1. Thus, the inability to respond to cytokine signaling may be an important consequence of epigenetic silencing.

The focus of Task 3 was to determine the impact of TMS1 loss on anchorage-independent growth potential. During tumor progression, epithelial cells acquire characteristics that are facilitated by lack of a normal response to apoptotic signals, including the ability to survive in the absence of substratum interactions, which would normally trigger an apoptotic response (termed "anoikis" or "detachment-induced apoptosis"). In normal breast tissue, TMS1 is selectively expressed in the ductal and lobular epithelium, but is absent from the underlying myoepithelium and stromal cells. This localization, combined with the proposed roles of TMS1 as a proapoptotic, tumor-suppressor molecule, suggests that TMS1 may play a role in the apoptotic response of epithelial cells to loss of substratum interactions. The ability to overcome apoptosis induced by loss of substratum interactions is an important step in tumorigenesis, and is one of the earliest events in establishing distant metastases. We originally proposed to study anchorage-independent growth potential by using cells stably knocked-down for TMS1 expression and comparing these cells to control cells for their ability to form colonies in soft agar over the course of three weeks. However, as we have had to use a transient siRNA transfection method to knock down TMS1 expression, we cannot perform the soft agar experiments, as the knockdown of TMS1 expression will only last for one week using our transient transfection method. As an alternative, we have optimized a system whereby cell culture dishes are coated with poly(2-hydroxyethyl-methacrylate), which is a hydrogel used to prevent adhesion of cells to growth surfaces. Seeding cells onto poly-HEMA plates triggers anoikis, thus allowing us to study anchorage-independent growth in a short-term setting. In recent experiments using this system, we found that cells lacking TMS1 expression showed an increase in the protein Bim, a BH3-only (Bcl-2 homology-3) protein required for anoikis (data not shown)[9]. The mechanism by which TMS1 loss leads to Bim upregulation, as well as the consequence of this upregulation, will be further examined.

C. Key Research Accomplishments

- Determined that loss of TMS1 causes resistance of breast cancer cells to anticancer agents and proapoptotic stimuli
  - Determined that TMS1-expressing MCF7 cells are less viable than TMS1 non-expressing cells following treatment with death receptor ligands or etoposide as seen by trypan blue exclusion
- Characterized the impact of TMS1 expression on death receptor-mediated apoptosis and NF-κB activity
  - Determined that TMS1 loss does not prevent caspase-8 activation following treatment with death-receptor ligands
  - Determined that TMS1 overexpression leads to caspase-8 activation in MCF7 cells
  - Determined that TMS1 loss does not impact NF-κB activity in response to death-receptor activation
- Characterized regulation of TMS1 by proapoptotic stimuli

- Determined that TNFα is a positive regulator of TMS1 expression in transformed and non-transformed breast epithelial cells, but not normal human fibroblasts
- Determined that the DNA-damaging agents etoposide and mitomycin c do not contribute to TMS1 expression, contrary to a previous report
- Identified the RelA/p65 subunit of NF-κB as a mediator of TNFα-induced upregulation of TMS1 in MCF7 breast epithelial cells
- Determined that neither secondary protein synthesis nor mRNA stability contribute to TNFα-induced upregulation of TMS1 in MCF7 breast epithelial cells
- Identified local chromatin alterations at the TMS1 locus following TNFα treatment in MCF7 breast epithelial cells
- Determined that DNaseI hypersensitivity sites found in TMS1 expressing breast epithelial cells are lacking in TMS1 non-expressing breast epithelial cells
- Determined that in a breast epithelial cell line in which TMS1 is silenced (MDA-MB231), treatment with TNFα or TRAIL does not restore TMS1 expression

- Preliminary characterization of the role of TMS1 in anoikis
  - Determined that loss of TMS1 expression leads to upregulation of Bim during anoikis

D. Reportable Outcomes

Awards:

- 2005 AACR Brigid G. Leventhal Women in Cancer Research Scholar Award

Abstracts:

- Parsons, M.J. and P.M. Vertino. Regulation of TMS1/ASC by Death Receptor Signaling in Breast Epithelial Cells (January 2005). AACR Special Conference Regulation of Cell Death in Oncogenesis, Waikoloa Village, HI.
- Parsons, M.J. and P.M. Vertino. Regulation of TMS1/ASC by Death Receptor Signaling in Breast Epithelial Cells (June 2005). Era of Hope 2005 Department of Defense Breast Cancer Research Program Meeting, Philadelphia, PA.

Presentations:

- Oral Presentation: Role of TMS1 in Death Receptor Signaling (October 2004). Graduate School Seminar. Emory University, Atlanta, GA
- Poster Presentation: Regulation of TMS1/ASC by Death Receptor Signaling in Breast Epithelial Cells (January 2005). AACR Special Conference: Regulation of Cell Death in Oncogenesis, Waikoloa Village, HI.
- Poster Presentation: Regulation of TMS1/ASC by Death Receptor Signaling in Breast Epithelial Cells (June 2005). Era of Hope 2005 Department of Defense Breast Cancer Research Program Meeting, Philadelphia, PA.

E. Conclusions

In summary, I have reported here that while TMS1 is not required for caspase-8 activation or NF-κB activity induced by death receptors, TMS1 overexpression leads to cleavage of caspase-8 in MCF7 cells, indicating that TMS1 may promote death-receptor induced apoptosis. Consistent with this idea, I have also shown that MCF7 cells lacking TMS1 expression via transient siRNA transfection are more resistant to apoptosis induced by death-receptor ligands or DNA damaging agents. In addition, I have examined the regulation of TMS1 by death-receptor signaling and proapoptotic stimuli. TMS1 is upregulated in response to TNFα at the level of transcription in breast epithelial cells, but not normal human diploid fibroblasts. This upregulation is dependent upon NF-κB and JNK activity, including the NF-κB subunit RelA/p65. The effects of TNFα are direct and do not involve secondary protein synthesis or mRNA stability. Consistent with this, TNFα treatment leads to local chromatin alterations at the TMS1 locus in MCF7 cells. DNaseI hypersensitive sites at the TMS1 locus that exist in the TMS1-expressing cell line MCF7 are lost in the cell line MDA-MB231, which is methylated and silenced at the endogenous TMS1 locus. Treatment of this cell line with TNFα or TRAIL is not sufficient to overcome this silencing, suggesting that upregulation of TMS1 by TNFα is dependent upon a particular chromatin structure. In addition, I have found that TMS1 loss leads to upregulation of the protein Bim in response to loss of substratum interactions. My work on this project in the last year has resulted in the submission of one manuscript (Appendix B), two abstracts, two poster presentations at national conferences, an oral presentation at my graduate university, and a Brigid G. Leventhal Women in Cancer Research scholar award from the American Association of Cancer Research. Over the next year, I will focus on completing Tasks 2 and 3 of my project, including extending my transient siRNA knockdown studies into other cell lines, including the non-transformed breast epithelial cell line MCF10A, to determine the effects of TMS1 loss on other transformed and pre-transformed cells.

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Appendix A.

Figure 1. Lack of TMS1 expression causes MCF7 cells to be resistant to death-receptor ligands and DNA damaging agents. *A*. MCF7 cells were transiently transfected with siRNA against TMS1 or lamin A/C as a control. 48 hours later cells were treated with death-receptor ligands or DNA damaging agents as indicated. After 24 hours, cells were trypsinized and viability was determined by trypan blue staining. Bars represent the mean of duplicate determinations and are represented as the fold increase in death compared to untreated cells. *B*. Protein lysates were prepared from the cells in *A* and examined by western blot analysis for TMS1 expression or GAPDH as a loading control.

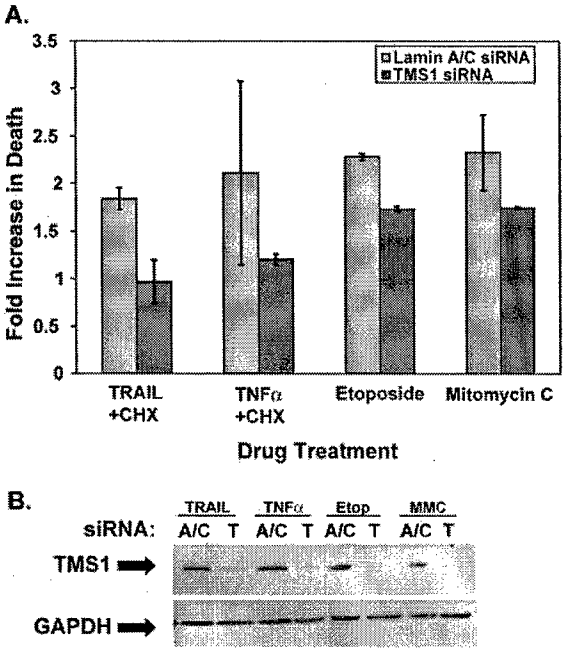


Figure 2. Impact of TMS1 downregulation on caspase-8 activation induced by TNFα or TRAIL. MCF7 cells were transfected with 200 nM siRNA targeted to TMS1 or lamin A/C. After 72 hours, cells were pretreated with 2 mg/ml cycloheximide for 30 minutes, followed by treatment with 30ng/ml TNFα (A) or 100ng/ml TRAIL (B) for 4 hr. Protein lysates were subjected to western blot analysis for caspase-8, TMS1 and either GAPDH or β-tubulin as indicated.

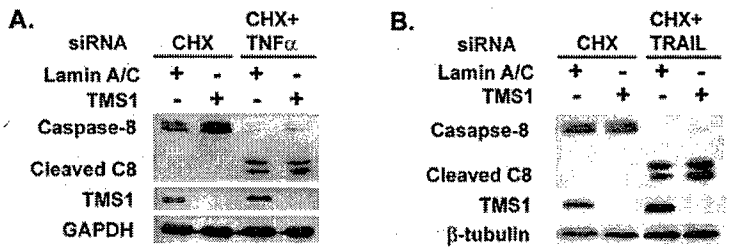


Figure 3. Overexpression of TMS1 in MCF7 cells induces cleavage of caspase-8. MCF7 cells were transfected with 1 μg empty vector (pcDNA3.1), or 1-2μg TMS1 expression construct (pcDNA-TMS1). Cell lysates were collected after 24 hr and subjected to western blot analysis using antibodies to TMS1, capsase-8, and β-tubulin as an internal loading control.

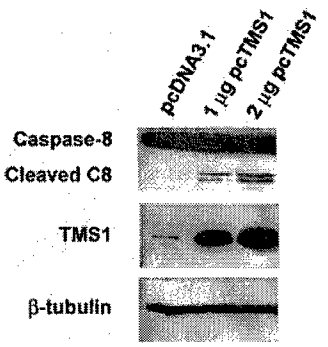


Figure 4. TMS1 is not required for activation of NF-κB by TNFα or TRAIL. MCF7 cells were transfected with 200 nM siRNA targeted to TMS1 or lamin A/C for 24h, followed by transfection with 200 ng of the NF-κB-responsive luciferase reporter plasmid, pNF-κB-Luc. Ten ng of a renilla luciferase control plasmid (pRL-TK) was included as a control for transfection efficiency. *A*, After 24h, cells were left untreated or treated with 30 ng/ml TNFα for an additional 48 hours, at which time luciferase activity was determined. Data represent the mean +/- standard deviation of triplicate determinations after correction for transfection efficiency. *B*, Total cellular protein collected from parallel cultures was analyzed for TMS1 and GAPDH by western blot analysis.

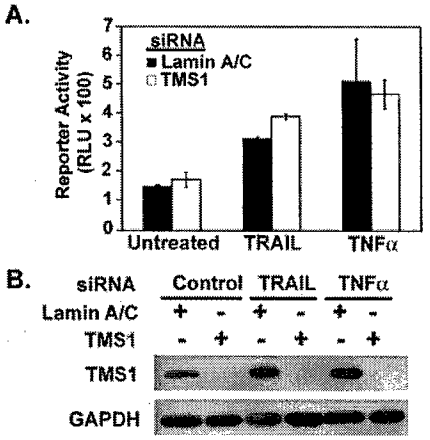


Figure 5. TNFα does not upregulate TMS1 expression in normal human diploid fibroblasts. *A*, Normal human diploid fibroblasts (IMR90) were treated over 48h with 30 ng/ml TNFα, and cell lysates were collected at the indicated time points. Total cellular proteins were subjected to western blot analysis using antibodies to TMS1 and β-tubulin as a loading control. *B*, IMR90 cells were treated as in *A*, and total cellular RNA was isolated at the indicated timepoints. TMS1 expression levels were quantified by reverse transcription and real-time PCR analysis. Data represent the levels of TMS1 mRNA normalized to that of an 18s rRNA internal control and are expressed relative to the value at time zero. Data represent the mean of duplicate PCR determinations which in general varied by less than 10%. Shown is a representative experiment which was repeated at least twice with similar results.

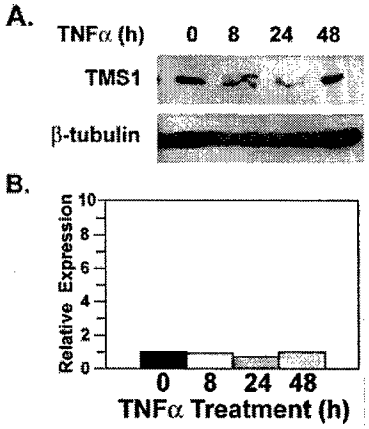


Figure 6. DNA damaging agents do not upregulate TMS1 expression in breast epithelial cells or normal human diploid fibroblasts. *A* and *B*, MCF7 breast cancer cells were treated with 50 μM etoposide (A) or 0.25 mg/ml mitomycin C (B) and cell lysates were collected at the indicated time points. Total cellular proteins were subjected to western blot analysis using antibodies to TMS1, p53 and p21 as indicated. β-tubulin served as a loading control. *C*, Normal human diploid fibroblasts (IMR90) were treated over 48h with 50 μM etoposide and analyzed for TMS1, p53 and p21 protein levels by western blot analysis as in panels *A* and *B*. *D-F*, MCF7 (D), MCF10A (E) and IMR90 (F) cells were treated with 50 uM etoposide and total RNA was isolated at the indicated timepoints. TMS1 expression levels were quantified by reverse transcription and real-time PCR analysis. Data represent the levels of TMS1 mRNA normalized to that of an 18s rRNA internal control and are expressed relative to the value at time zero. Data represent the mean of duplicate PCR determinations which in general varied by less than 10%.

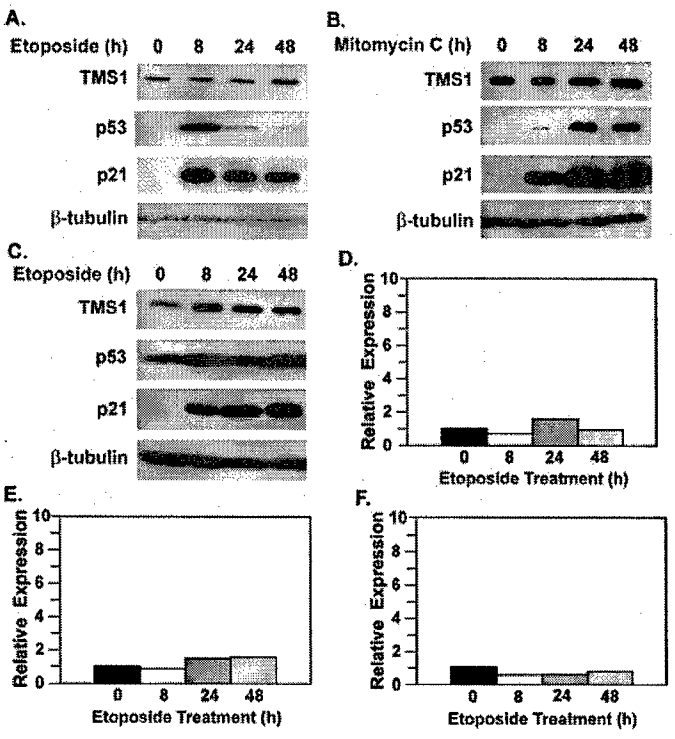


Figure 7. p65/RelA is required for TNFα-induced upregulation of TMS1 in MCF7 cells. . *A and B*, MCF7 cells were transfected with 200 nM siRNA targeted to p65/RelA or lamin A/C. After 24 hours, cells were left untreated or treated with 30 ng/ml TNFα for an additional 24 hours. *A*, TMS1 mRNA expression was determined by real time RT-PCR. Data represent the mean of duplicate PCR determinations which varied by less than 10%. Shown is a representative of three independent experiments. *B*, Protein lysates from parallel cultures were fractionated by SDS-PAGE and subjected to western blot analysis using antibodies to TMS1, p65/RelA and GAPDH.

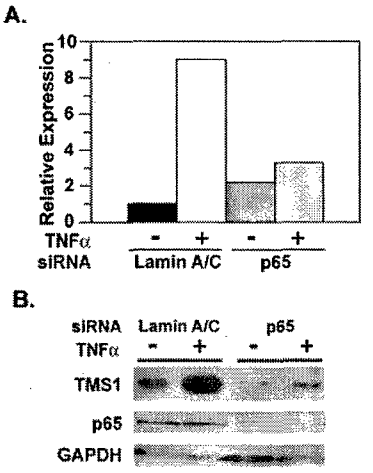


Figure 8. Secondary protein synthesis and mRNA stability are not required for TNFα-induced upregulation of TMS1. *A and B*, MCF7 cells were left untreated or pretreated with 0.025 μg/ml or 2 μg/ml cycloheximide for 1 hr, followed by 30 ng/ml TNFα for 48 hr. TMS1 mRNA and protein expression were analyzed by real-time RT-PCR (*A*) and western blot analysis (*B*) as described above. Shown is a representative of two independent experiments. *C*, MCF7 cells were pretreated for 30 minutes with 1 μg/ml actinomycin D, followed by the addition of 0 or 30 ng/ml TNFα. Total RNA was isolated at the indicated times and analyzed for TMS1 mRNA levels by real-time RT-PCR as described above. TMS1 levels are expressed relative to the value at time zero after normalization to the levels of an 18S rRNA internal control. Data represent the mean of duplicate PCR determinations which varied by less than 10%.

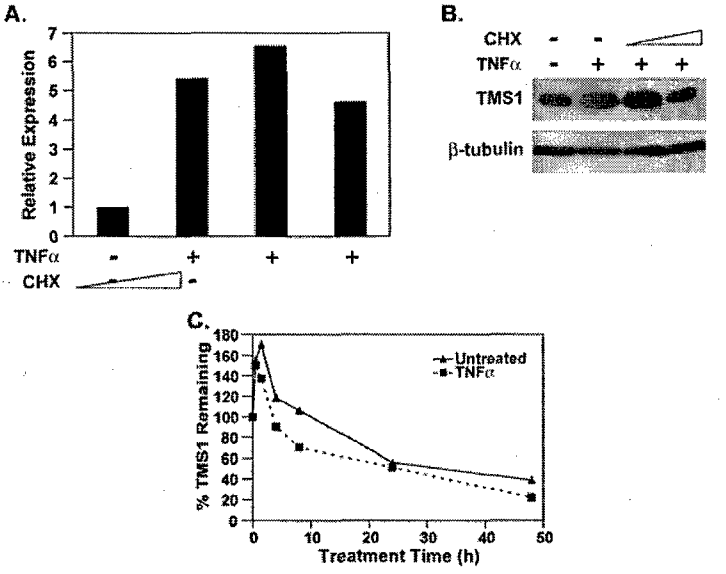
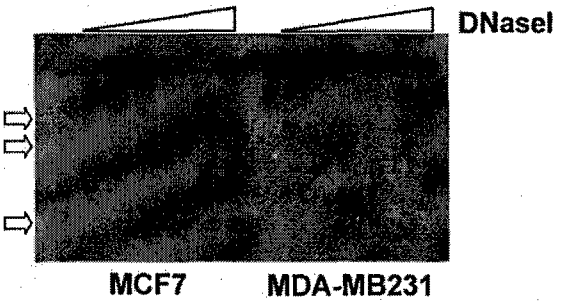


Figure 9. DNaseI Hypersensitive Site Analysis of MCF7 and MDA-MB231 Breast Epithelial Cells. Nuclei were isolated from MCF7 and MDA-MB231 breast epithelial cells and digested with increasing amounts of DNaseI. DNA was isolated and subjected to Southern blot analysis with a radiolabelled probe anchored to the 3' end of the TMS1 locus. Arrows indicate DNaseI hypersensitive sites.



Appendix B

Dual Role of TMS1 in Death-Receptor Signaling

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Abstract

Aberrant DNA methylation of promoter region CpG islands is associated with gene silencing and serves as an alternative to mutations in the inactivation of tumor suppressor genes in human cancers. We identified a gene TMS1 (for Target of Methylation-mediated Silencing) that is subject to such epigenetic silencing in a significant proportion of human breast and other cancers. Also known as ASC, TMS1 encodes a bipartite intracellular signaling molecule with proposed roles in apoptosis and inflammation. However, the precise role of this protein in the pathogenesis of breast and other cancers has not been clearly defined. In this study, we examined the role of TMS1 in death receptor signaling. We found that TMS1 is upregulated in response to treatment with TRAIL and TNFα in breast epithelial cells, but not in human fibroblasts. This upregulation was not dependent on new protein synthesis or alterations in mRNA stability, suggesting a direct effect on TMS1 transcription. Induction of TMS1 by TNFα was blocked by coexpression of a dominant negative IκBα, siRNA-mediated knockdown of RelA/p65, or concurrent treatment with SP600125, indicating a requirement for the NF-κB and JNK signaling pathways. Although previous work has suggested that TMS1 may be directly regulated by p53, we found that whereas treatment of breast epithelial cells or normal diploid fibroblasts with DNA damaging agents resulted in the stabilization of endogenous p53 and a concomitant increase in p21, it had little impact on the expression of TMS1 mRNA or protein. We further show that whereas TMS1 is not required for TNFα or TRAIL-induced activation of NF-κB or caspase-8, it can promote caspase-8 activation independently of death receptor-ligand interactions. Taken together, these data suggest that upregulation of TMS1 by TNFα and subsequent activation of caspase-8 could function to amplify the apoptotic signal induced by death receptors in some cell types, including breast epithelial cells. Epigenetic silencing of TMS1 in breast cancer may therefore dampen the cellular response to cytokine signaling.

Introduction

Acquired genetic alterations which lead to defects in apoptosis are common in human cancers and are thought to promote tumorigenesis by allowing cells to survive under conditions that would otherwise trigger a cell death response. Unlike normal cells, cancer cells survive despite DNA damage, in the absence of required growth factors and under the hypoxic conditions encountered when a tumor outgrows its blood supply. To metastasize, tumor cells must survive transit through the blood stream and colonize a foreign tissue, though normal epithelial cells die when deprived of substratum interactions. Importantly, the propensity of tumor cells to undergo apoptosis is a critical determinant of their sensitivity to many chemotherapeutic agents. Therefore, defects in apoptotic signaling contribute to tumor initiation and progression, and can lead to drug resistance and treatment failure.

Gene silencing associated with aberrant methylation of CpG island-containing gene promoters serves as an alternative, epigenetic mechanism contributing to loss of gene function in human cancers. CpG islands are CpG dense regions that flank the 5' end of more than half the genes in the human genome. Though normally maintained in an unmethylated state, CpG islands can become aberrantly methylated in cancer. This methylation is accompanied by a shift to a repressed chromatin conformation which renders the associated gene transcriptionally silent. Once established, this state is mitotically heritable and contributes to the stable silencing of tumor suppressor and other genes. Several proapoptotic genes succumb to epigenetic silencing in human tumors, including caspase-8, the TRAIL receptors DR4 and DR5, the caspase-9 adaptor Apaf-1, and the death-associated protein kinase, DAPK (Esteller et al., 2001; Furukawa et al., 2005; Horak et al., 2005; Soengas et al., 2001; Teitz et al., 2000; van Noesel et al., 2002), suggesting that acquired epigenetic alterations also contribute to apoptotic resistance during tumor progression.

In a screen for targets of methylation-mediated silencing, we identified a gene TMS1 (for Target of Methylation-mediated Silencing), that is methylated and silenced in a significant proportion of human breast cancers (Conway et al., 2000). Subsequent studies by our lab and others have implicated the epigenetic silencing of TMS1 in a number of other tumor types, including melanomas, glioblastomas, non small cell lung cancers, gastric, and colorectal cancers (Guan et al., 2003; Moriai et al., 2002; Stone et al., 2004; Virmani et al., 2003; Yokoyama et al., 2003). Ectopic expression of TMS1 suppresses the growth of breast cancer cells, consistent with a role in tumor suppression (Conway et al., 2000).

At present, the precise function of TMS1 and the consequences of its silencing during carcinogenesis are not known. Also known as ASC and PYCARD, TMS1 encodes a bipartite adaptor protein containing an N-terminal pyrin domain (PYD) and a C-terminal caspase recruitment domain (CARD), two members of the death-domain superfamily of protein-protein interaction domains. These domains are often found in proteins that participate in apoptosis and inflammation, and there is evidence supporting a role for TMS1 in both of these processes. Evidence from overexpression studies indicates that TMS1 can drive apoptosis in a caspase 9-dependent manner (McConnell & Vertino, 2000). Furthermore, TMS1 binds to the Bcl-2 family member Bax and facilitates its translocation to the mitochondrion in response to cytotoxic agents (Ohtsuka et al., 2004). Consistent with a role in the intrinsic (mitochondrial) cell death pathway, antisense-mediated knockdown of TMS1 protects cells from apoptosis induced by cytotoxic agents (Masumoto et al., 1999). TMS1 has also been implicated in the extrinsic apoptotic pathway, as it binds to and stimulates cleavage of caspase-8, the apical caspase in apoptosis induced by death receptors (Masumoto et al., 2003). Recent biochemical data and evidence from knockout mice indicate that TMS1 functions as an adaptor for caspase-1, and that it is necessary for the maturation of proinflammatory cytokines in response to bacterial pathogens in cells of

myeloid lineage (Mariathasan et al., 2004). Whether it plays a similar, or perhaps additional role in epithelial cells is not known.

Currently, the natural upstream stimulus for TMS1-dependent apoptosis is not well defined. Furthermore, the regulation of TMS1 has not been extensively studied in breast epithelial cells, which upon transformation often undergo a loss of TMS1 expression though epigenetic silencing. In this study we examined the role of TMS1 in the cellular response to initiators of the extrinsic (death receptor) and intrinsic cell death pathways in breast epithelial cell lines and normal diploid fibroblasts. We find here that TMS1 is induced in breast epithelial cells in response to the death receptor ligands TRAIL and TNFα. The induction by TNFα is specific to epithelial cells and is dependent on both NF-κB and JNK/AP-1 signaling. Although a previous report has suggested that TMS1 is regulated by p53 (Ohtsuka et al., 2004), we found little impact of DNA damaging agents on TMS1 expression in this study. Furthermore, we show that TMS1 is not required for NF-κB or caspase-8 activation induced by death-receptor ligands in MCF7 cells, but TMS1 promotes cleavage and activation of caspase-8 when overexpressed. These data indicate that TMS1 is both regulated by and promotes death-receptor signaling in breast epithelial cells. Epigenetic silencing of TMS1 may therefore contribute to carcinogenesis by dampening the cellular response to cytokines.

Materials and Methods:

Cell Lines, Drug Treatments and Reagents. MCF7 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM (4.5. g/liter glucose) plus 10% FCS and 2 mM glutamine. MCF10A cells were obtained from the Karmanos Cancer Institute (Detroit, MI) and maintained in DMEM/F12 plus 5% FCS, 20 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin, and 2 mM glutamine. IMR90 normal human diploid fibroblasts were obtained from the National Institute on Aging Cell Repository and maintained in EMEM with 10% FCS and 2 mM glutamine. Where indicated, cells were treated with 50 μM etoposide (Sigma), 0.25 mg/ml mitomycin c (Sigma), 100 ng/ml 6x his-tagged recombinant human TRAIL (R&D Systems) plus 1 μg/ml 6X polyhistidine crosslinking antibody (R&D Systems), or 30 ng/ml recombinant human TNFα (R&D Systems). The JNK inhibitor SP600125 was purchased from Biomol. The dominant-negative IκBα adenovirus (Ad-mIκBα) was a gift from Dr. Leland Chung (Emory University). Actinomycin D and cycloheximide were purchased from Sigma. For plasmid transfections, MCF7 cells (4.0×10^5) were seeded in 6-well plates and transfected the following day with 1 ug pcDNA-3.1+ or 1-2 ug pcTMS1 using Lipofectamine according to the manufacturer's instructions. Protein lysates were collected 24 hours following transfection.

Immunoblotting and Antibodies. Cells were pelleted, washed three times in 1X PBS, and lysed with RIPA buffer containing 1X protease inhibitors (Complete Mini Protease Inhibitor Cocktail, Roche), 1mM orthovanadate, and 10 mM sodium fluoride (Sigma). Total protein (100 μg) was separated on a 15% SDS-PAGE gel, transferred to PVDF (BioRad), and probed with the indicated primary antibody. Immuno complexes were detected by incubation with HRP-

conjugated secondary antibody and chemiluminescence detection (Pierce). The antibodies used were: anti-ASC (MBL), β-tubulin (Sigma), p53 (AB-7, Oncogene), p21 (Ab-1, Oncogene), c-jun (Cell Signaling Technologies), phosphorylated-c-jun (Ser 63, Cell Signaling Technologies), IκBα (Cell Signaling Technologies), phospho-IκBα (Ser 32/36, Cell Signaling Technologies), caspase-8 (1C12 – Cell Signaling Technologies), NF-κB p65 (Santa Cruz), and GAPDH (Abcam).

Reverse Transcription and Real-Time PCR. Total RNA was isolated using the RNeasy Mini Kit according to the manufacturer's directions (Qiagen). Total RNA was treated with amplification grade DNase I (Invitrogen) and reverse-transcribed (RT) using random hexamer priming and MMLV-reverse transcriptase (Invitrogen). One μl of a 50X dilution of the RT reaction was amplified in duplicate using the iQ SYBR Green Supermix kit (BioRad) and the MyIQ real-time detection system. Reaction conditions included a hot start (3 min, 95°C), followed by 50 cycles of (95°C, 10 s; 55°C, 60 s). Melt curve analysis was performed to ensure a single product species. Parallel reactions were performed using primers to 18S rRNA as an internal control. Relative starting quantities were calculated by comparison to a common standard curve generated with a dilution series of MCF10A cell cDNA that was included in each run. Primers for real-time PCR analysis were for TMS1, 5'-TCC AGC AGC CAC TCA ACG-3' and 5'-GCA CTT TAT AGA CCA GCA -3'; and for 18S, 5'-GAG GGA GCC TGA GAA ACG G-3' and 5'-GTC GGG AGT GGG TAA TTT GC-3'.

Luciferase Reporter Assays. A genomic *Hind*III-*Nco*I fragment containing 1254 bp upstream of the TMS1 translation start codon was cloned into the pGL3 luciferase reporter plasmid (Stratagene) to form the construct pTMS1-1254-Luc. Cells (8.0×10^4) were seeded in 24-well

plates and transfected the next day with 200 ng pTMS1-1254-Luc or 200 ng of an NF-κB luciferase reporter construct (pNF-κB-Luc, Stratagene) using 0.6 μl of FuGene6 (Roche) per well. A Renilla luciferase reporter (10 ng of pRL-TK, Promega) was included as a control for transfection efficiency. After 24 hours, medium on the transfected cells was replaced with fresh media or media containing 30 ng/ml TNFα or 100 ng/ml 6x his-tagged recombinant human TRAIL plus 1 μg/ml 6X polyhistidine crosslinking antibody as indicated. After 8 hours of treatment, cells were lysed and firefly luciferase and renilla luciferase activities were determined using the Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer's directions.

siRNA Experiments: MCF7 cells (2.0×10^5) were seeded in six-well plates and transfected the next day with 200 nM of the indicated siRNA using Oligofectamine (Invitrogen) according to the manufacturer's instructions. siRNA duplexes were purchased from Dharmacon (Lafeyette, CO). The sequence of the TMS1 siRNA was: 5'-CGA GGG UCA CAA ACG UUG A dTdT-3' (sense), and the sequence of the p65 siRNA was: 5'-GCC CUA UCC CUU UAC GUC A dTdT-3' (sense). Dharmacon lamin A/C siControl was used to control for non-specific effects of siRNA transfection.

Results:

TMS1 is normally highly expressed in immune cells, particularly neutrophils and cells of the macrophage/monocyte lineage. Previous studies have shown that TMS1/ASC is induced in these cell types in response to pro-inflammatory stimuli including IL-1β, LPS and TNFα (Shiohara et al., 2002; Stehlik et al., 2003). TMS1 is also expressed in many epithelial cell types (Masumoto et al., 2001). In normal breast tissue, TMS1 is selectively expressed in the ductal and lobular epithelium, but is absent from the underlying myoepithelium and stromal cells (McConnell & Vertino, 2004). At present, little is known about the factors that normally regulate TMS1 expression, and the function of this protein in cells of non-immune origin has not been extensively studied.

To gain insight into the regulation of TMS1 in inflammatory and apoptotic processes, we characterized the response of normal and neoplastic breast epithelial cells to death-receptor ligands and DNA damaging agents. Treatment of the breast cancer cell line MCF7 with TNFα caused a significant increase in TMS1 protein expression (Figure 1a). TRAIL also induced upregulation of TMS1 protein, although to a lesser extent and with delayed kinetics relative to TNFα (Figure 1a). In contrast, treatment with mitomycin c resulted in a modest upregulation of TMS1 at later timepoints, while etoposide had no effect. That treatment with etoposide and mitomycin c elicited a normal p53-dependent DNA damage response in this cell type was confirmed by an observed stabilization of endogenous p53 and a concomitant upregulation of the p53 target gene p21 (Figure 1a).

To determine if the effects of these agents on TMS1 expression were particular to epithelial cells, we also examined the impact of death receptors and DNA damaging agents on TMS1 protein expression in IMR90 normal diploid fibroblasts. Unlike breast epithelial cells, TRAIL and TNFα had little effect on the expression of TMS1 in fibroblasts (Figure 1b). Thus,

the upregulation of TMS1 by TNFα and TRAIL is cell-type dependent. As observed for MCF7 cells, etoposide induced stabilization of endogenous p53 and upregulation of p21, but had only a modest impact on TMS1 levels (Figure 1b).

The impact of death-receptor signaling and DNA damage on TMS1 expression was further examined by quantitative real-time reverse transcriptase PCR for TMS1 message. Consistent with the observed upregulation of TMS1 protein, treatment of MCF7 cells with TNFα or TRAIL resulted in a time-dependent, five-fold and two-fold increase in TMS1 mRNA, respectively, after 48 hours (Figure 2a). In contrast, treatment with etoposide had little impact on TMS1 mRNA levels (Figure 2a). Consistent with the modest upregulation observed at the protein level, mitomycin c treatment resulted in a 2-fold increase in TMS1 mRNA levels after 48 hours (Figure 2a). The upregulation of TMS1 by TNFα was not a consequence of transformation, as TNFα treatment resulted in a similar nine-fold upregulation of TMS1 in the immortalized, non-transformed breast epithelial cell line MCF10A (Figure 2b). Consistent with the findings in MCF7 cells, etoposide treatment had no effect on TMS1 mRNA expression in MCF10A cells (Figure 2b). In IMR90 fibroblasts, neither TNFα nor etoposide had any impact on TMS1 mRNA levels (Figure 2c). Taken together, these data indicate that TNFα upregulates TMS1 mRNA and protein expression in breast epithelial cells, but not human fibroblasts, whereas agents that induce DNA damage have little effect on TMS1 regulation in either cell type.

We next investigated the mechanism of TNFα-induced upregulation of TMS1. The interaction of TNFα with its membrane receptors TNFRI and II initiates downstream signaling through the jun kinase (JNK) pathway and the IKK pathway, culminating in activation of the transcription factors AP-1 (fos and jun) and NF-κB, respectively (Devin et al., 2000; Natoli et al., 1997; Wajant et al., 2003). To determine whether activation of JNK plays a role in death-

receptor induced upregulation of TMS1, MCF7 cells were treated with TNFα in the presence of the JNK inhibitor SP600125 (Bennett et al., 2001). Inhibition of JNK signaling blocked the TNFα-induced upregulation of TMS1 mRNA and protein in a dose-dependent manner (Figures 3a and 3b). To control for the efficiency of JNK inhibition by SP600125, parallel samples were also analyzed for c-jun and phosphorylated c-jun (Ser 63) protein levels (Figure 3b). Cellular levels of c-jun are autoregulated in that activation of JNK results in phosphorylation of c-jun and ATF-2, which leads to transactivation of the c-jun promoter (Shaulian & Karin, 2002). At concentrations of SP600125 that were sufficient to block TNFα-induced phosphorylation and upregulation of c-jun, upregulation of TMS1 was also blocked (Figure 3b). These results indicate that TNFα-induced TMS1 upregulation is mediated at least in part by JNK signaling.

To determine if TNFα-induced activation of NF-κB contributes to TMS1 upregulation, MCF7 cells were treated with TNFα in the absence or presence of an adenoviral vector expressing a dominant-negative form of IκBα (Ad-mIκBα, S32A, S36A) (Mayo et al., 2003). The IκBα super-repressor contains alanines instead of serines at positions 32 and 36, and as a consequence cannot be phosphorylated by the IKK complex or degraded by the 26S proteasome (Brockman et al., 1995; Mayo et al., 2003). Expression of the dominant-negative IκBα thus sequesters NF-κB in the cytoplasm, thereby preventing NF-κB dependent transcription (Brockman et al., 1995; Mayo et al., 2003). The expression of IκBα super-repressor blocked TNFα-induced upregulation of TMS1 mRNA and protein in a dose-dependent manner, indicating that intact NF-κB signaling is required for TNFα-induced upregulation of TMS1 (Figure 3c and 3d). As shown in Figure 3d, TNFα induced the phosphorylation and degradation of endogenous IκBα, confirming that the NF-κB pathway was activated. As expected, the

stabilized dominant-negative IκBα mutant was unaffected by TNFα treatment and activation of the NF-κB pathway (Figure 3d).

Classic NF-κB is a heterodimer comprised of the p65/RelA and p50 subunits (Baeuerle & Baltimore, 1996; Baldwin, 1996). As a further test for the requirement of NF-κB, we examined the impact of siRNA-mediated knockdown of p65/RelA on TNFα-induced upregulation of TMS1. Knockdown of p65/RelA expression also blocked TNFα-induced upregulation of TMS1 mRNA and protein (Figures 3e and f), suggesting that NF-κB containing the p65/RelA subunit is acting to promote the TNFα-induced upregulation of TMS1. Taken together, these results indicate that both JNK and NF-κB signaling contribute to the TNFα-induced upregulation of TMS1.

NK-κB and JNK activity are known to stimulate the rapid expression of a number of proinflammatory and antiapoptotic genes, some of which could themselves be contributing to the TNFα-induced upregulation of TMS1. The time-frame of TMS1 induction following TNFα stimulation, which was not observed until 24 hours after treatment (Figures 1 and 2), is also suggestive of the involvement of a TNFα-regulated intermediate. To address this possibility, MCF7 cells were treated with TNFα in the absence or presence of cycloheximide to block new protein synthesis. At concentrations sufficient to block new protein synthesis (as indicated by a reduction of TMS1 protein), cycloheximide had no impact on TNFα-induced upregulation of TMS1 mRNA (Figures 4a and 4b). These data indicate that protein synthesis is not required for TNFα-induced upregulation of TMS1, ruling out the involvement of a TNFα-regulated intermediate.

Taken together, the above data are most consistent with a direct effect of TNFα on TMS1 transcription. To test this, reporter studies were performed in which a construct containing a

1254 bp TMS1 promoter construct driving a luciferase reporter gene (Levine et al., 2003) was transfected into MCF7 cells, and the response to TNFα was determined. A reporter construct containing five tandem copies of a consensus NF-κB response element driving luciferase was also used as a positive control. Although TNFα induced an eight-fold increase in luciferase expression from the consensus NF-κB reporter, it had no effect on TMS1 promoter-driven reporter activity (Figure 4c).

Alternatively, TNFα could impact TMS1 expression levels through changes in mRNA stability. To test this possibility, MCF7 cells were pretreated with actinomycin D to inhibit transcription, followed by treatment with TNFα. In the absence of TNFα, the half-life of the TMS1 message was approximately 24 hours (Figure 4d). The stability of TMS1 mRNA was not significantly altered by treatment of cells with TNFα, indicating that changes in mRNA stability do not contribute to the TNFα-induced upregulation of TMS1.

The above studies indicate that TMS1 is upregulated in response to death receptor stimuli and suggest that TMS1 may play a role in some of the downstream events induced by TNFα-mediated signaling pathways. As discussed above, TNFα signaling induces the activities of the NF-κB and JNK signaling pathways. This is achieved through binding of TNFα to the TNFα receptor (TNFR), which in turn recruits TRADD and RIP or TRAF2 to induce activation of NF-κB (Kelliher et al., 1998; Natoli et al., 1997) and JNK (Yeh et al., 1997), respectively. Previous reports have suggested that TMS1 can either promote or inhibit NF-κB signaling depending on cell type, the coexpression of specific adaptor proteins and/or TMS1 expression levels (Grenier et al., 2002; Manji et al., 2002; Stehlik et al., 2002; Wang et al., 2002). The vast majority of these studies have utilized ectopic expression of TMS1. To examine the involvement of endogenous TMS1 in NF-κB signaling, we determined the impact of TMS1 knockdown on

TNFα or TRAIL-induced activation of NF-κB in MCF7 cells. siRNA-mediated knockdown of TMS1 had no effect on activation of NF-κB in response to TRAIL or TNFα (Figure 5a and 5b). These data indicate that TMS1 is not required for TNFα- or TRAIL-induced activation of NF-κB in breast epithelial cells.

In addition to stimulating NF-κB and JNK signaling, TNFα and TRAIL can induce apoptosis. In this scenario, TNF-bound TNFRI recruits the adaptor protein FADD via homophilic death-domain interactions to form the death-inducing signaling complex (DISC) (Ashkenazi & Dixit, 1998; Chinnaiyan et al., 1996; Hsu et al., 1996). The subsequent recruitment of caspase-8 to the DISC allows for its activation via induced proximity, thereby stimulating capsase-8 dependent apoptosis. Previous work has suggested that TMS1 binds to and activates capsase-8 (Hasegawa et al., 2005; Masumoto et al., 2003). Therefore the upregulation of TMS1 by TNFα and TRAIL may serve to promote caspase-8 dependent apoptosis. To test the involvement of TMS1 in death receptor-induced apoptosis, MCF7 cells were treated with TRAIL or TNFα in the presence of siRNA against TMS1. siRNA-mediated knockdown of TMS1 had little impact on TNFα- or TRAIL-induced cleavage and activation of caspase-8, indicating that TMS1 is dispensable for casase-8 activation induced by death receptors in breast epithelial cells (Figures 5c and 5d),. We next determined the effect of TMS1 overexpression on caspase-8 cleavage in MCF7 cells. Western analysis illustrated that TMS1 overexpression induced caspase-8 cleavage in a dose-dependent manner (Figure 5e). Taken together, these data indicate that TMS1 is not required for death-receptor induced activation of caspase-8, but is sufficient to induce cleavage of caspase-8 independent of a death receptor stimulus. Thus, the upregulation of TMS1 following death-receptor signaling may function as an apoptotic feedback look that amplifies the apoptotic stimulus induced by TRAIL and TNFα.

Discussion:

TMS1 is a novel tumor suppressor gene that is subject to frequent epigenetic silencing in several different tumor types, including breast, gliomas, melanomas and non small cell lung cancers, however the mechanism by which TMS1 silencing promotes carcinogenesis remains unclear (Conway et al., 2000; Guan et al., 2003; Moriai et al., 2002; Virmani et al., 2003; Yokoyama et al., 2003). Although it has been previously reported that TMS1 is upregulated by cytokines such as IL-1β, IFN-γ, TNFα and LPS in immune cells (Shiohara et al., 2002; Stehlik et al., 2003), the regulation of TMS1 in cells of non-myeloid origin is poorly understood. Here we examined the role of TMS1 in the cellular response of breast epithelial cells and human fibroblasts to death receptors and DNA damaging agents. We find that, in both transformed and non-transformed breast epithelial cells, TMS1 is upregulated in response to TNFα, and to a lesser extent TRAIL. This response was cell-type dependent and did not occur in normal diploid fibroblasts. In contrast, DNA damaging agents that elicit a p53-dependent apoptotic response have little impact on TMS1 expression in either cell type. Human diploid fibroblasts did not upregulate TMS1 in response to either TNFα or etoposide, indicating that the induction of TMS1 expression following death receptor activation is specific to epithelial cells.

TNFα is a cytokine which, upon binding, induces receptor trimerization and recruitment of death domain-containing adaptor proteins to the receptor's cytoplasmic death-domain. Recruitment of TRADD, RIP, and TRAF2 to the cytoplasmic TNFR death domains form submembranous protein complexes that activate downstream signaling through the NF-κB and JNK/AP-1 pathways (Kelliher et al., 1998; Lin et al., 2000; Yeh et al., 1997). The upregulation of TMS1 by TNFα treatment is dependent upon the activities of both of these signaling pathways, and does not involve secondary protein synthesis or increased mRNA stability, suggesting that the effect of TNFα on TMS1 is direct. However, there was no effect of TNFα on

a TMS1 reporter construct in transient transfection assays (Figure 4c). There are several potential explanations for these findings. First, TNFα-induced upregulation of TMS1 could require *cis* elements distal to the promoter that are not present in the reporter construct. This idea is supported by the fact that there are putative AP-1 and NF-κB sites both upstream and downstream of the TMS1 transcription start site (data not shown). It is also possible that the regulation of TMS1 by TNFα requires a specific chromatin environment not represented in the transiently transfected reporter construct. Consistent with these ideas, we have observed that TNFα-treatment induces local alterations in chromatin structure at the TMS1 locus in by DNase-I hypersensitive site analysis (data not shown).

NF-κB and JNK signaling often results in upregulation of target genes involved in anti-apoptotic and inflammatory pathways, thus, the upregulation of TMS1 by TNFα in an NF-κB and JNK-dependent manner suggests that TMS1 may be involved in downstream signaling events related to anti-apoptosis and inflammation. Indeed, one proposed role for TMS1 is in the regulation of NF-κB itself. NF-κB signaling is important for proper immune function and is often dysregulated in cancer. Studies addressing the impact of TMS1 on NF-κB activity are largely conflicting and have shown that TMS1 can promote or inhibit NF-κB activity depending on the stimulus and the co-expression of other adaptor proteins such as the PYPAFs or members of the pyrin family (Grenier et al., 2002; Manji et al., 2002; Stehlik et al., 2002; Wang et al., 2002)}. For example, it has been reported by multiple groups that co-expression of TMS1 with the adaptor proteins PYPAF1/cryopyrin, PYPAF5 or PYPAF7 induces NF-κB activity (Grenier et al., 2002; Manji et al., 2002; Stehlik et al., 2002; Wang et al., 2002), although overexpression of either full-length TMS1 or the pyrin domain of TMS1 alone inhibits NF-κB activity induced by TNFα, Bcl-10 or Nod-1 (Stehlik et al., 2002). Most of these studies utilize overexpression of

TMS1 or forced interactions between TMS1 and other adaptor proteins which may or may not be physiologically relevant. To this end, a recent study by Mariathasan *et. al.* (Mariathasan et al., 2004) showed that TMS1 is dispensable for IκBα degradation, ERK1/2 activation and NF-κB activation in response to LPS and TNFα in macrophages. In addition, it was recently published that cryopyrin does not induce NF-κB activity alone or in the presence of TMS1 in 293 cells stably expressing physiologic levels of TMS1 (Yu et al., 2005). Similarly, we find here that complete knockdown of TMS1 in breast epithelial cells has no effect on NF-κB activity induced by TNFα or TRAIL. Thus, it seems that TMS1 is not necessary for activation of NF-κB. Whether it functions in a feedback mechanism to inhibit NF-κB remains to be determined. However, contrary to the report discussed above, experiments from our lab indicate that high level expression of TMS1 in 293 cells does not affect TNFα-induced activation of NF-κB (data not shown).

Although NF-κB and JNK signaling is thought to elicit an anti-apoptotic response, there is also evidence to suggest that these pathways are also necessary for apoptosis in some circumstances. For example, JNK knockout mice are resistant to UV-induced apoptosis (Tournier et al., 2000). Likewise, c-jun -/- cells are resistant to apoptosis and instead undergo cell-cycle arrest in response to UV damage (Shaulian & Karin, 2002; Shaulian et al., 2000). Also, Fas ligand is upregulated by NF-κB and AP-1 following T-cell activation, and the subsequent Fas-mediated cell death is dependent upon NF-κB (Kasibhatla et al., 1998; Kasibhatla et al., 1999). TMS1 may be another example of an NF-κB/JNK-regulated protein that functions in a proapoptotic capacity. Previous work in our lab shows that TMS1 induces apoptosis when overexpressed in 293 cells (McConnell & Vertino, 2000). Moreover, recent studies show that TMS1 is necessary for translocation of Bax to the mitochondria following

DNA damage (Ohtsuka et al., 2004). Thus, upregulation of TMS1 by NF-κB and JNK signaling following TNFα stimulation may serve to promote apoptosis rather than to inhibit it.

In this paper we addressed this possibility by examining the requirement of TMS1 for caspase-8 cleavage and activation induced by TRAIL and TNFα. Our siRNA experiments indicate that TMS1 is not required for -mediated knockdown of TMS1 had no impact on caspase-8 cleavage and activation induced by death-receptors. However, TMS1 overexpression is still capable of promoting caspase-8 cleavage. Thus, it is possible that the upregulation of TMS1 following by TNFα and TRAIL in epithelial cells, while not required for initial activation of caspase-8, may serve to amplify the apoptotic signal induced by death-receptors through a direct interaction with caspase-8. There is mounting evidence that there exists a cytoplasmic pool of caspase-8 that can be activated independently of the DISC. Following death-receptor stimulation, it is thought that caspase-8 is recruited to the death-inducing signaling complex (DISC) by adaptor proteins such as FADD and TRADD (Ashkenazi & Dixit, 1998; Sharp et al., 2005) allowing for activation of the procaspase through induced proximity(Salvesen & Dixit, 1999; Shi, 2004). There are a number of studies showing that chemotherapeutic agents can induce capsase-8 cleavage and activation independently of DISC formation. Ferreira *et. al.* showed that the drugs cisplatin, topotecan and gemcitabine all induced caspase-8 activation and apoptosis in non-small cell lung cancer cells, and that this was not blocked by overexpression of a dominant-negative FADD or two different inhibitors of caspase-9, indicating that these drugs are activating caspase-8 independently of the DISC complex and the mitochondrial apoptotic pathway (Ferreira et al., 2000). Similarly, Masumoto *et. al.* (Masumoto et al., 2003) illustrated that TMS1-induced caspase-8 activation and apoptosis cannot be blocked with a dominant-negative FADD, which suggests that TMS1 is also capable of activating caspase-8 independent of death receptor-ligand interactions. Thus, the possibility exists that upregulation of TMS1

following stimulation of cells with TNFα could function to enhance the apoptotic signal through direct activation of caspase-8.

Recently it was reported that TMS1 is a direct downstream target of p53 induced by genotoxic stress in human fibroblasts and breast epithelial cells (Ohtsuka et al., 2004). These authors showed that overexpression of p53 resulted in upregulation of TMS1. They also showed that TMS1 is upregulated following mitomycin c and ionizing radiation in IMR90 cells and, to a lesser degree, in MCF7 cells, and that this increase could be blocked by siRNA-mediated knockdown of p53. The authors thus propose a direct regulation of TMS1 by p53. Here we find that such agents have a relatively minor impact on TMS1 expression despite an intact p53 response, as indicated by robust upregulation of p21 and stabilization of p53. Similar results were obtained in all cell types tested, including MCF7 cells, MCF10A cells, and IMR90 fibroblasts. Moreover, intact TMS1 promoter constructs in transient transfection assays do not respond to etoposide when transfected into MCF7 or IMR90 cells despite the presence of a putative p53 site (data not shown). Thus, whereas stimuli that induce p53 may have some impact on expression of TMS1, the effect may not be direct.

In summary, we have shown here that TMS1 is upregulated by TRAIL and TNFα in breast epithelial cells in an NF-κB and JNK-dependent manner. While TMS1 is not required for the initial activation of caspase-8 following death-receptor stimulation, overexpression of TMS1 can promote caspase-8 activation. Therefore, the upregulation of TMS1 by death-receptor signaling in breast epithelial cells may function to enhance the apoptotic signal. One consequence of epigenetic silencing of TMS1 in breast and other cancers may be an attenuated response to death-receptor mediated apoptosis induced by TNFα and TRAIL, thereby conferring a survival advantage to cancer cells. Indeed, TNFα and TRAIL have no impact on TMS1 expression in breast cancer cell lines where the endogenous gene is methylated, such as MDA-

MB231 cells (unpublished observations). The inability to respond to cytokine signaling is thus an important consequence of epigenetic silencing.

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Figure Legends:

Figure 1. TMS1 is upregulated in response to TNFα and TRAIL, but not DNA damaging agents. *A*, MCF7 breast cancer cells were treated with 30 ng/ml TNFα, 100 ng/ml TRAIL, 50 μM etoposide, or 0.25 mg/ml mitomycin C and cell lysates were collected at the indicated time points. Total cellular proteins were subjected to western blot analysis using antibodies to TMS1, p53 and p21 as indicated. β-tubulin levels were similarly analyzed and served as a loading control. *B*, Normal human diploid fibroblasts (IMR90) were treated over 48h with 50 μM etoposide, 100 ng/ml TRAIL or 30 ng/ml TNFα, and analyzed for TMS1, p53 and p21 protein levels by western blot analysis as in panel *A*.

Figure 2. Effect of death receptor ligands and DNA damaging agents on the expression of TMS1 mRNA. *A*, MCF7 cells were treated with 30 ng/ml TNFα, 100 ng/ml TRAIL, 0.25 mg/ml mitomycin c, or 50 μM etoposide, and total cellular RNA was isolated at the indicated timepoints. TMS1 expression levels were quantified by reverse transcription and real-time PCR analysis. Data represent the levels of TMS1 mRNA normalized to that of an 18s rRNA internal control and are expressed relative to the value at time zero. Data represent the mean of duplicate PCR determinations which in general varied by less than 10%. Shown is a representative experiment which was repeated at least twice with similar results. *B*, MCF10A breast epithelial cells were treated with 30 ng/ml TNFα or 50 μM etoposide over 48h and analyzed for TMS1 expression as in *A*. *C*, IMR90 fibroblasts were treated with 50 μM etoposide or 30 ng/ml TNFα and analyzed for TMS1 mRNA expression as in *A*. TMS1 expression levels at time zero differed between the cell lines such that IMR90 cells and MCF10A cells express approximately 10-20 fold less TMS1 than MCF7 cells.

Figure 3. AP-1/JNK and NF-κB signaling are required for the induction of TMS1 by TNFα. *A and B*, MCF7 cells were either left untreated or were pretreated with 2 μM, 10 μM or 25 μM SP600125 for 30 min. TNFα was then added to a final concentration of 30 ng/ml for 48h. *A*, TMS1 mRNA expression was determined by real time RT-PCR as described in Figure 2. Data represent the mean of duplicate PCR determinations which in general varied by less than 10%. Shown is a representative of two independent experiments with similar results. *B*, MCF7 cells were treated as described in *A*. Protein lysates were fractionated by SDS-PAGE and subjected to western blot analysis using antibodies to TMS1, phospho-c-jun, c-jun, and β-tubulin as indicated. *C and D,* MCF7 cells were infected with 0, 20 or 100 m.o.i. of an adenoviral construct expressing dominant-negative IκBα (Ad-mIκBα). After 18 hr, cells were left untreated or treated with 30 ng/ml TNFα and incubated for an additional 48 hr. *C*, TMS1 mRNA expression was determined by real time RT-PCR. Data represent the mean of duplicate PCR determinations which in general varied by less than 10%. Shown is a representative experiment. *D*, Protein lysates from parallel cultures were fractionated by SDS-PAGE and subjected to western blot analysis using antibodies to TMS1, IκBα, phospho-IκBα, and β-tubulin. *E and F*, MCF7 cells were transfected with 200 nM siRNA targeted to p65/RelA or lamin A/C. After 24 hours, cells were left untreated or treated with 30 ng/ml TNFα for an additional 24 hours. *E*, TMS1 mRNA expression was determined by real time RT-PCR. Data represent the mean of duplicate PCR determinations which varied by less than 10%. Shown is a representative of three independent experiments. *F*, Protein lysates from parallel cultures were fractionated by SDS-PAGE and subjected to western blot analysis using antibodies to TMS1, p65/RelA and GAPDH.

Figure 4. *A and B*, New protein synthesis is not required for TNFα-induced upregulation of TMS1. MCF7 cells were left untreated or pretreated with 0.025 μg/ml or 2 μg/ml

cycloheximide for 1 hr, followed by 30 ng/ml TNFα for 48 hr. TMS1 mRNA and protein expression were analyzed by real-time RT-PCR (*A*) and western blot analysis (*B*) as described in Figures 1-3. Shown is a representative of two independent experiments. ***C*, Effect of TNFα on the TMS1 promoter.** MCF7 cells were transfected with 200 ng of an NF-κB-responsive luciferase reporter plasmid (pNF-κB-Luc, Stratagene) or a TMS1 promoter luciferase reporter plasmid (pTMS1-1254-Luc) using the FuGene reagent. Ten ng of a renilla luciferase plasmid (pRL-TK) was included as a control for transfection efficiency. After 18 hr, cells were left untreated or treated with 30 ng/ml TNFα for 48 hr, at which time luciferase activity was determined. Data represent the mean +/- standard deviation of triplicate determinations after correction for transfection efficiency. ***D*, Effect of TNFα on TMS1 message stability.** MCF7 cells were pretreated for 30 minutes with 1 μg/ml actinomycin D, followed by the addition of 0 or 30 ng/ml TNFα. Total RNA was isolated at the indicated times and analyzed for TMS1 mRNA levels by real-time RT-PCR as described in Figure 2. TMS1 levels are expressed relative to the value at time zero after normalization to the levels of an 18S rRNA internal control. Data represent the mean of duplicate PCR determinations which varied by less than 10%.

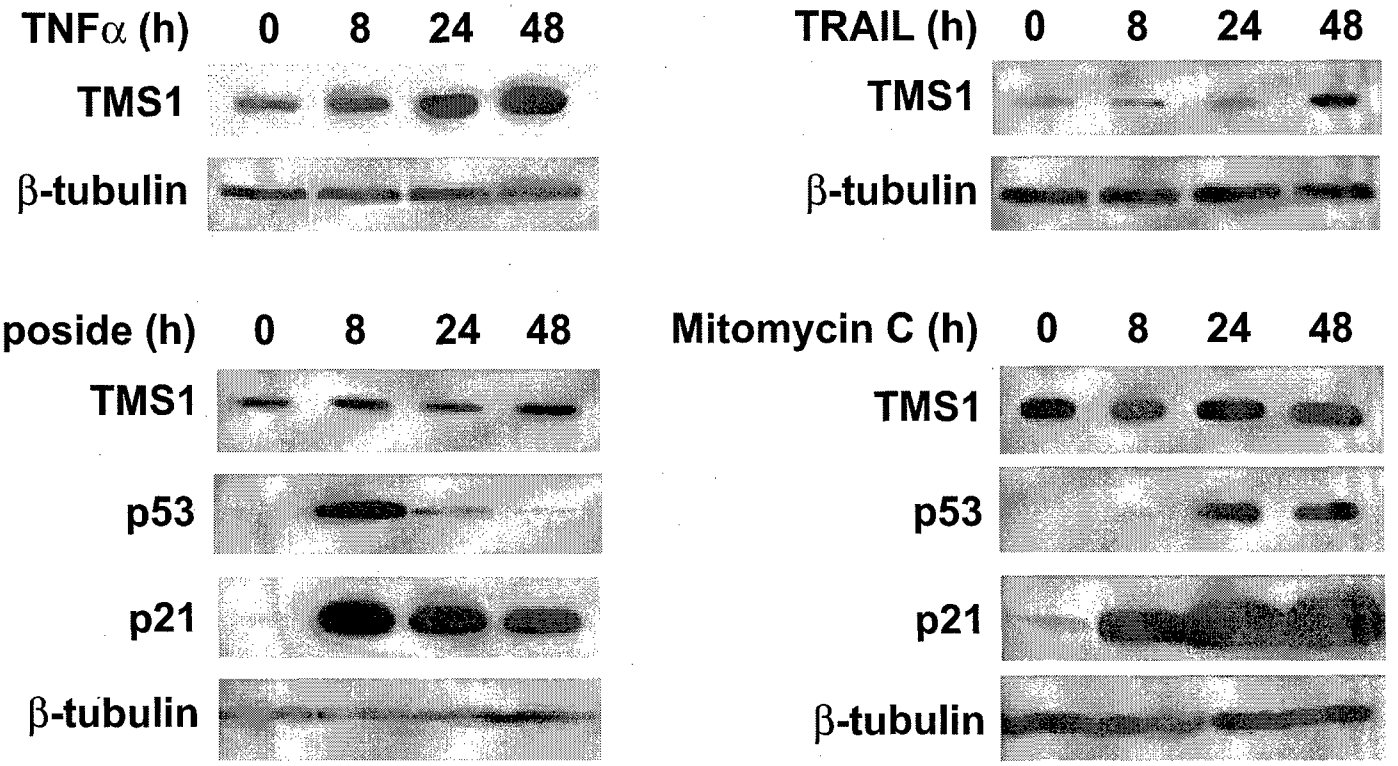
Figure 5. Effect of TMS1 on TNFα and TRAIL-induced activation of NF-κB or the extrinsic cell death pathway.

A and B, TMS1 is not required for activation of NF-κB by TNFα or TRAIL. MCF7 cells were transfected with 200 nM siRNA targeted to TMS1 or lamin A/C for 24h, followed by transfection with 200 ng of the NF-κB-responsive luciferase reporter plasmid, pNF-κB-Luc. Ten ng of a renilla luciferase control plasmid (pRL-TK) was included as a control for transfection efficiency. *A*, After 24h, cells were left untreated or treated with 30 ng/ml TNFα for an

additional 48 hours, at which time luciferase activity was determined. Data represent the mean +/- standard deviation of triplicate determinations after correction for transfection efficiency. *B*, Total cellular protein collected from parallel cultures was analyzed for TMS1, caspase-8 and GAPDH by western blot analysis. *C* and *D*, Impact of TMS1 downregulation on caspase-8 activation induced by TNFα or TRAIL. MCF7 cells were transfected with 200 nM siRNA targeted to TMS1 or lamin A/C. After 72 hours, cells were pretreated with 2 mg/ml cycloheximide for 30 minutes, followed by treatment with 30ng/ml TNFα (B) or 100ng/ml TRAIL (C) for 4 hr. Protein lysates were subjected to western blot analysis for caspase-8, TMS1 and either GAPDH or β-tubulin as indicated. *D*, Overexpression of TMS1 in MCF7 cells induces cleavage of caspase-8. MCF7 cells were transfected with 1 μg empty vector (pcDNA3.1), or 1-2μg TMS1 expression construct (pcDNA-TMS1). Cell lysates were collected after 24 hr and subjected to western blot analysis using antibodies to TMS1, capsase-8, and β-tubulin as an internal loading control.

Figure 1

A.


TNFα (h) 0 8 24 48
TMS1
β-tubulin
TRAIL (h) 0 8 24 48
TMS1
β-tubulin
Etoposide (h) 0 8 24 48
TMS1
p53
p21
β-tubulin
Mitomycin C (h) 0 8 24 48
TMS1
p53
p21
β-tubulin

B.

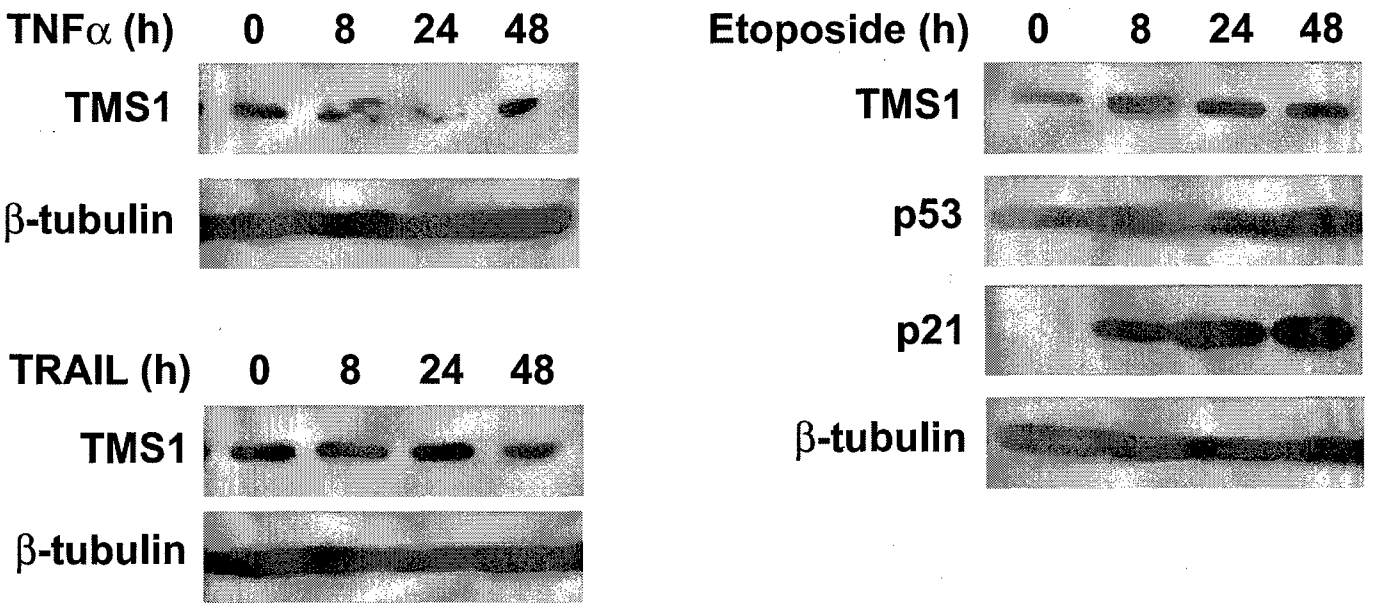
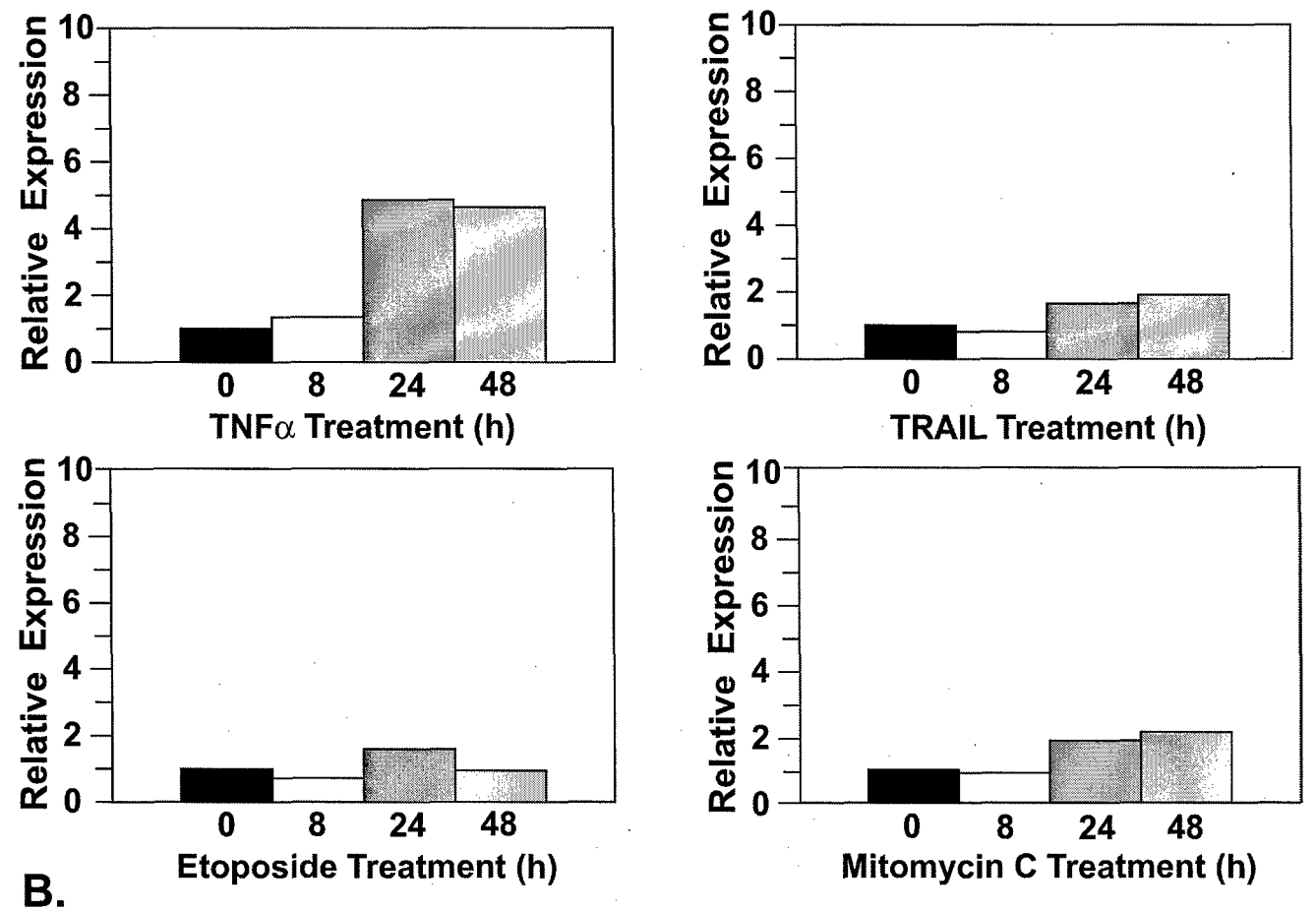
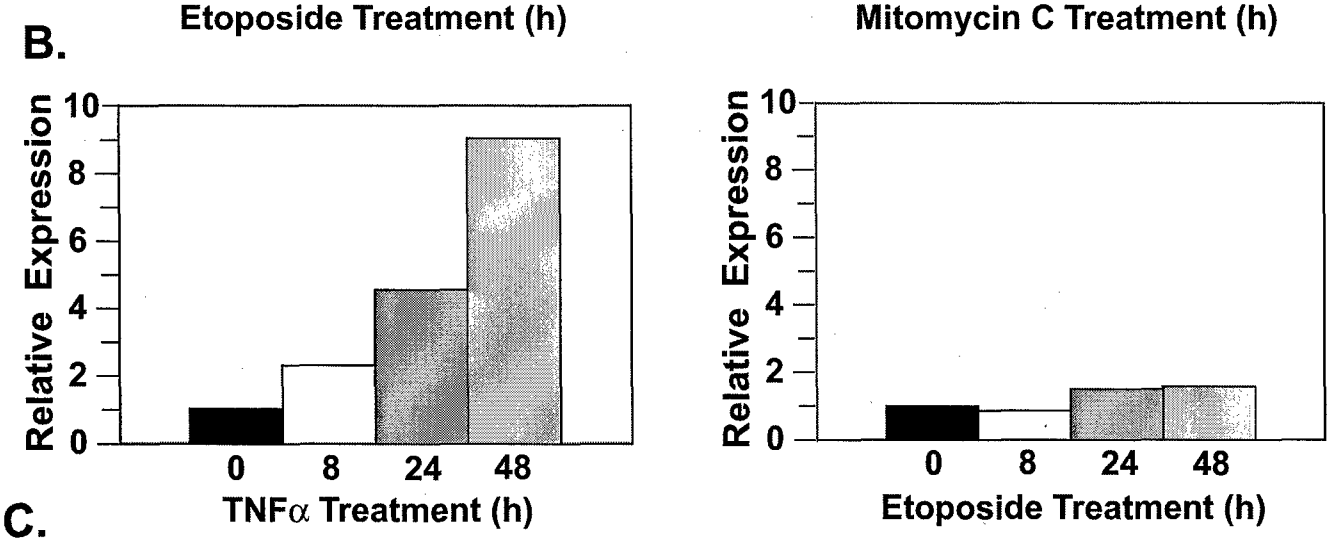

TNFα (h) 0 8 24 48
TMS1
β-tubulin
TRAIL (h) 0 8 24 48
TMS1
β-tubulin
Etoposide (h) 0 8 24 48
TMS1
p53
p21
β-tubulin

Figure 2


A.
Relative Expression
0
2
4
6
8
10
0
8
24
48
TNFα Treatment (h)
TRAIL Treatment (h)
Etoposide Treatment (h)
Mitomycin C Treatment (h)


B.
Relative Expression
0
2
4
6
8
10
0
8
24
48
TNFα Treatment (h)
Etoposide Treatment (h)

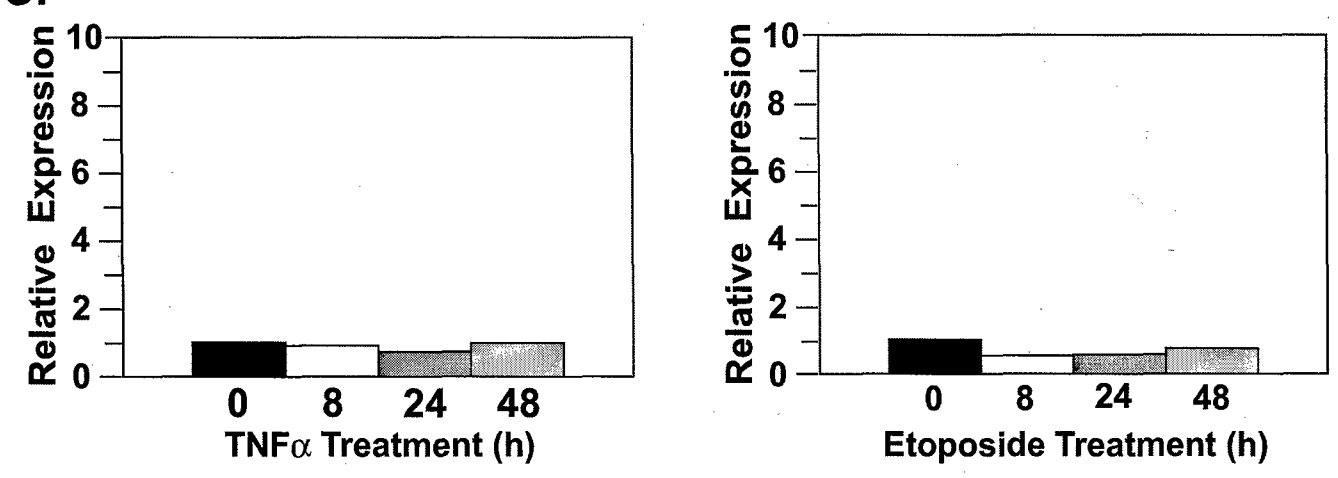

C.
Relative Expression
0
2
4
6
8
10
0
8
24
48
TNFα Treatment (h)
Etoposide Treatment (h)

Figure 3

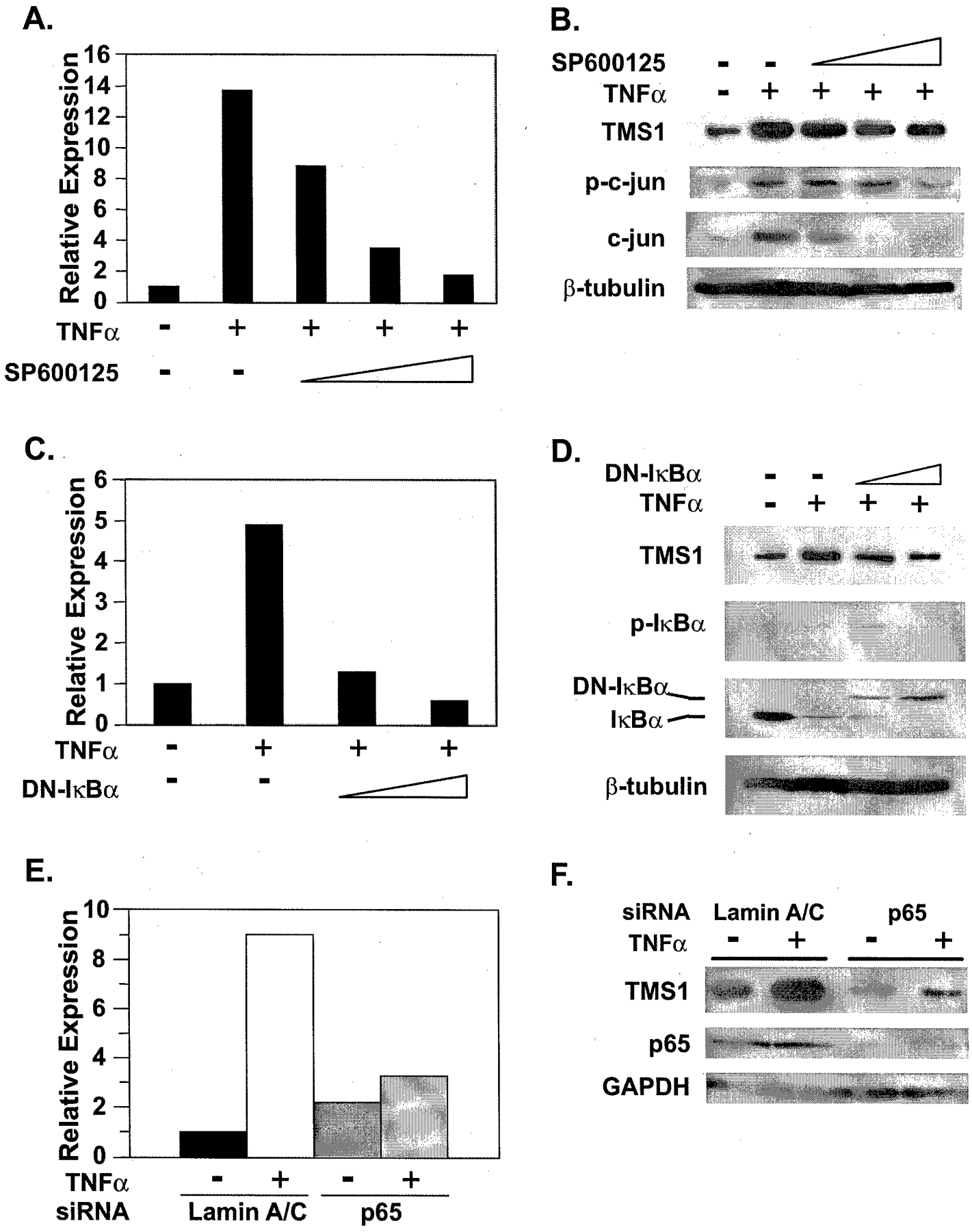

A.
Relative Expression
0
2
4
6
8
10
12
14
16
TNFα - + + + +
SP600125 - - + + +
B.
SP600125 - - + + +
TNFα - + + + +
TMS1
p-c-jun
c-jun
β-tubulin
C.
Relative Expression
0
1
2
3
4
5
6
TNFα - + + +
DN-IκBα - - + + +
D.
DN-IκBα - - + + +
TNFα - + + +
TMS1
p-IκBα
DN-IκBα
IκBα
β-tubulin
E.
Relative Expression
0
2
4
6
8
10
TNFα - + - +
siRNA Lamin A/C p65
F.
siRNA Lamin A/C p65
TNFα - + - +
TMS1
p65
GAPDH

Figure 4

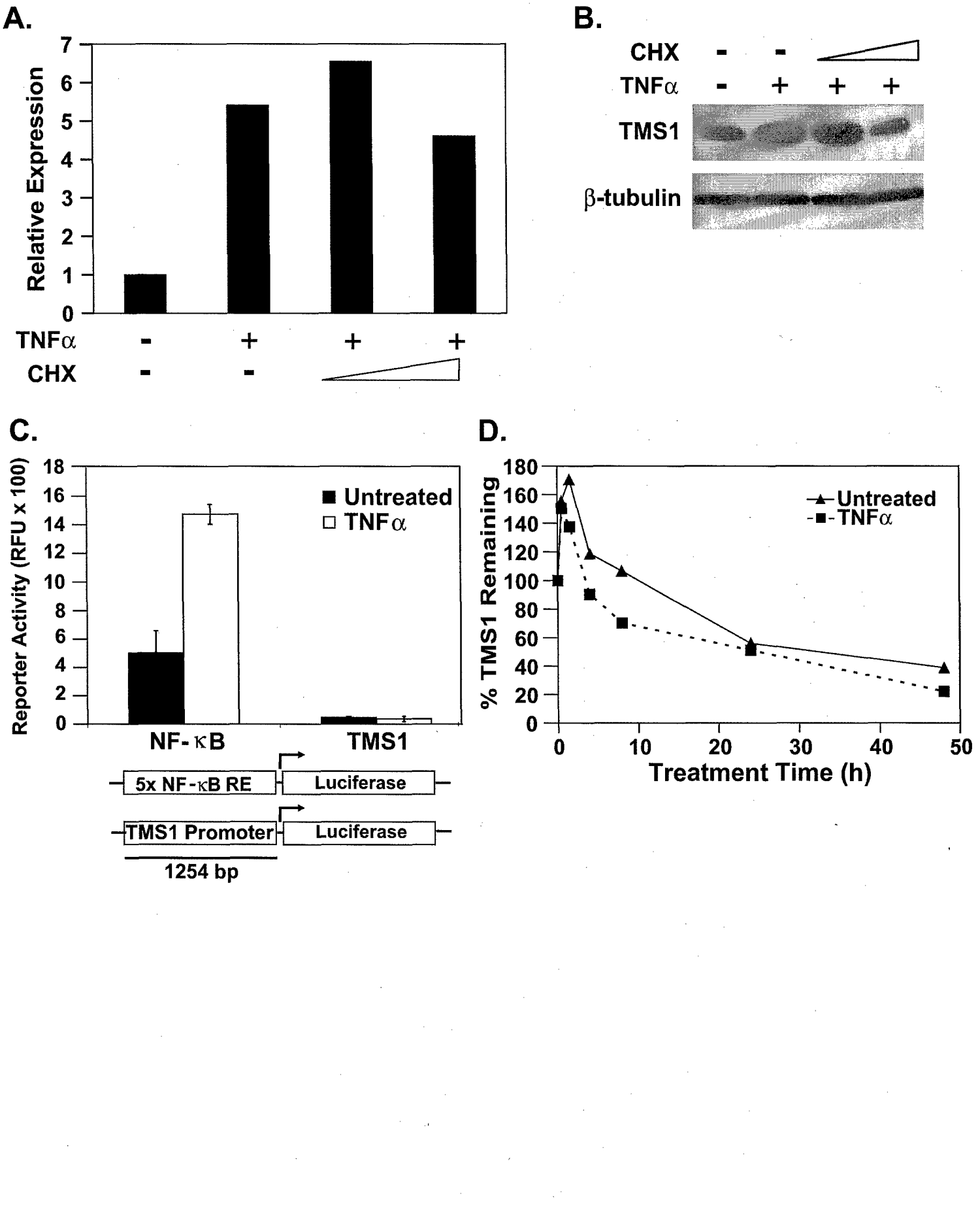
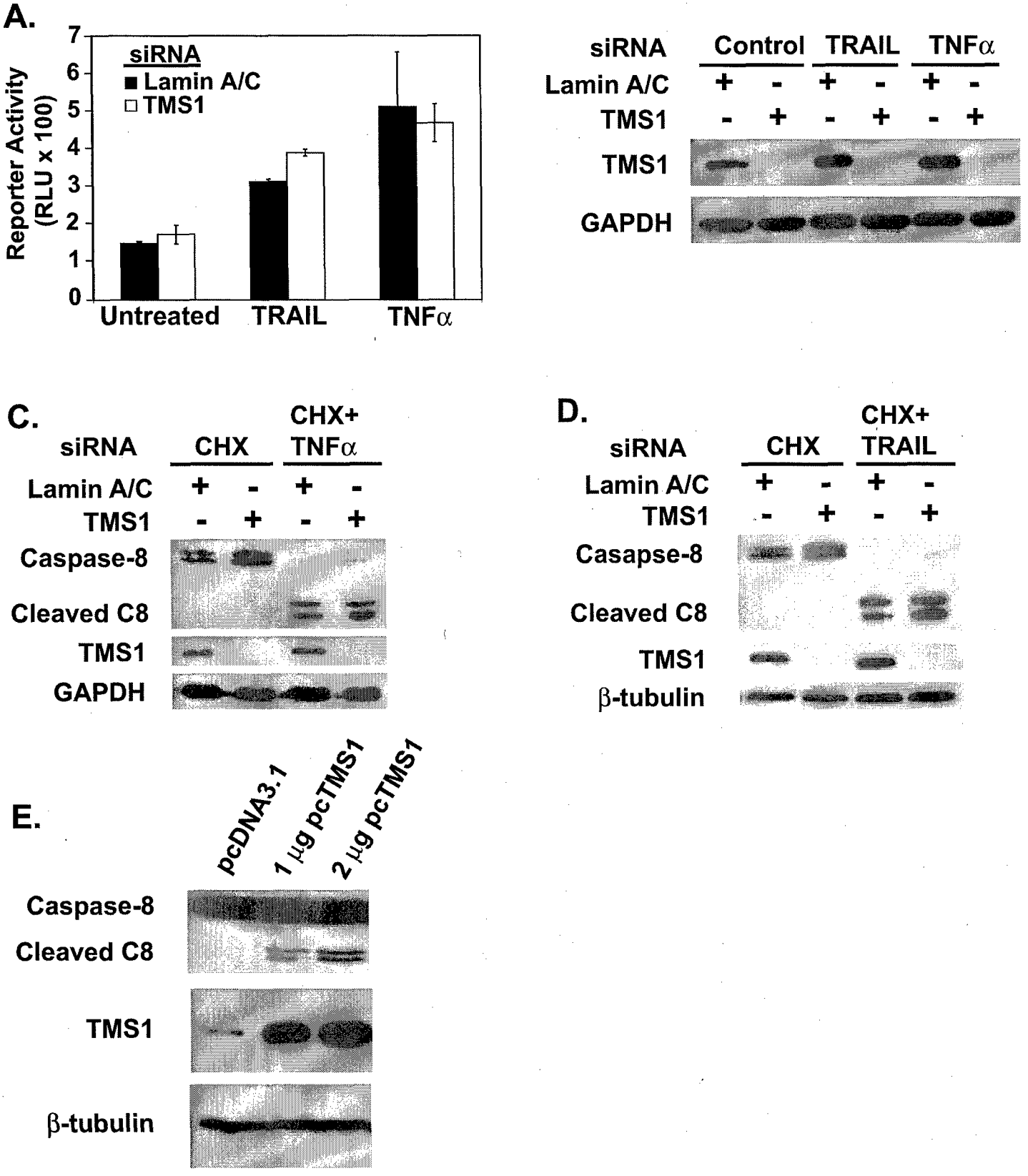

A.
Relative Expression
0
1
2
3
4
5
6
7
TNFα - + + +
CHX - - ▲
B.
CHX - - ▲
TNFα - + + +
TMS1
β-tubulin
C.
Reporter Activity (RFU x 100)
0
2
4
6
8
10
12
14
16
18
Untreated
TNFα
NF-κB
TMS1
5x NF-κB RE
Luciferase
TMS1 Promoter
Luciferase
1254 bp
D.
% TMS1 Remaining
0
20
40
60
80
100
120
140
160
180
Untreated
TNFα
0
10
20
30
40
50
Treatment Time (h)

Figure 5


A.
siRNA
Lamin A/C
TMS1
Reporter Activity (RLU x 100)
0
1
2
3
4
5
6
7
Untreated
TRAIL
TNFα
B.
siRNA
Control
TRAIL
TNFα
Lamin A/C
TMS1
GAPDH
C.
siRNA
CHX
CHX+ TNFα
Lamin A/C
TMS1
Caspase-8
Cleaved C8
TMS1
GAPDH
D.
siRNA
CHX
CHX+ TRAIL
Lamin A/C
TMS1
Casapse-8
Cleaved C8
TMS1
β-tubulin
E.
pcDNA3.1
1 μg pcTMS1
2 μg pcTMS1
Caspase-8
Cleaved C8
TMS1
β-tubulin